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Sphingosine Phosphate Lyase Expression Is Essential for Normal Development in *Caenorhabditis elegans**

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Sphingolipids are ubiquitous membrane constituents whose metabolites function as signaling molecules in eukaryotic cells. Sphingosine 1-phosphate, a key sphingolipid second messenger, regulates proliferation, motility, invasiveness, and programmed cell death. These effects of sphingosine 1-phosphate and similar phosphorylated sphingoid bases have been observed in organisms as diverse as yeast and humans. Intracellular levels of sphingosine 1-phosphate are tightly regulated by the actions of sphingosine kinase, which is responsible for its synthesis and sphingosine-1-phosphate phosphatase and sphingosine phosphate lyase, the two enzymes responsible for its catabolism. In this study, we describe the cloning of the *Caenorhabditis elegans* sphingosine phosphate lyase gene along with its functional expression in *Saccharomyces cerevisiae*. Promoter analysis indicates tissue-specific and developmental regulation of sphingosine phosphate lyase gene expression. Inhibition of *C. elegans* sphingosine phosphate lyase expression by RNA interference causes accumulation of phosphorylated and unphosphorylated long-chain bases and leads to poor feeding, delayed growth, reproductive abnormalities, and intestinal damage similar to the effects seen with exposure to *Bacillus thuringiensis* toxin. Our results show that sphingosine phosphate lyase is an essential gene in *C. elegans* and suggest that the sphingolipid degradative pathway plays a conserved role in regulating animal development.

Cell proliferation, migration, and apoptosis are critical to embryogenesis. Elucidating the signal transduction pathways that regulate these cellular processes can lead to insights into normal animal development and the pathophysiology of developmental diseases. Sphingosine 1-phosphate (S1P)[1] is an endogenous sphingolipid metabolite that regulates mammalian cell proliferation, apoptosis, and migration (1, 2). S1P is unique in its ability to function in two signaling capacities, first as an intracellular second messenger and second as a ligand for a subset of G protein-coupled cell surface receptors of the endothelial differentiation gene (Edg) family (3, 4). Additionally, there is substantial evidence to support the existence of a "sphingolipid rheostat" whereby cell fate decisions are determined by the ratio of intracellular levels of S1P, a known proliferative stimulus, and ceramide, an inducer of apoptosis (5).

Intracellular levels of S1P are controlled by its synthesis, catabolism, and export to the extracellular space. Sphingosine kinase (SK) catalyzes the phosphorylation of sphingosine, generating S1P (6). SK activity increases in response to a variety of inducers, including fetal calf serum, platelet-derived growth factor, nerve growth factor, muscarinic acetylcholine agonists, tumor necrosis factor α, and cross-linking of the FcγRI and FcϵRI immunoglobulin receptors (7–13). Sphingosine phosphate lyase (SPL) catalyzes the cleavage of S1P at the C_{2-3} carbon bond to yield a long-chain aldehyde and ethanolamine phosphate (14). SPL is a pyridoxal 5′-phosphate-dependent member of the carbon-carbon lyase subclass of aldehydelyases. The reaction proceeds optimally at pH 7.4–7.6, requires chelators due to sensitivity toward heavy metal ions, and is irreversible (15). SPL activity is ubiquitous in mammalian tissues, with the notable exception of platelets, in which SPL activity is absent. Although modulation of S1P levels is considered the major biological function of this enzyme, the products of the reaction can contribute to synthesis of both fatty acids and phospholipids and have been shown to regulate sterol regulatory element binding protein processing in *Drosophila melanogaster*, indicating that the effects of this enzyme on lipid metabolism and biology may be broader than previously appreciated (16). SPL was first identified through a genetic screen for sphingosine resistance in *Saccharomyces cerevisiae* (17). Subsequently, functional SPL homologs of *Mus musculus* and *Homo sapiens* were cloned and characterized (18, 19). Furthermore, predicted protein sequences demonstrating significant homology to known SPL proteins in *D. melanogaster*, *Neurospora crassa*, *Dictyostelium discoideum*, *Danio rerio*, and *Arabidopsis thalliensis* have been identified, indicating that SPL may be highly conserved. In addition to its degradation by SPL, S1P can be dephosphorylated back to sphingosine. Enzymes capable of catalyzing the dephosphorylation of S1P comprise two groups, the S1P phosphatases, which demonstrate sphingoid base substrate specificity, and a family of lipid phosphatases capable of dephosphorylating various substrates, including lysophosphatidic acid, S1P, and phosphatidic acid (20–22).

Knowledge of SPL, SK, and S1P phosphatase gene sequences from a variety of genetically tractable organisms has made possible the use of both forward and reverse genetic approaches to elucidate the role of sphingolipid metabolism in animal development. Recent studies have implicated roles for both S1P and SPL in the developmental processes of different species, including slime mold, zebrafish, and mouse (23–25).

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[1] The abbreviations used are: S1P, sphingosine 1-phosphate; *DPL1*, dihydrosphingosine phosphate lyase; ds, double-stranded; Edg, endothelial differentiation gene; GFP, green fluorescent protein; IPTG, isopropyl β-1-thiogalactopyranoside; RNAi, RNA interference; SK, sphingosine kinase; SPL, sphingosine phosphate lyase; S1PP, sphingosine-1-phosphate phosphatase; YEPD, yeast extract, peptone, and dextrose.

TABLE I
Primers used in these experiments

Primer name	Primer sequence
5′-B0222.4	5′-GATCGAATTCACAGAATAAAATGGATTTTGCACTGGAGC-3′
3′-B0222.4	5′-GACTGAATTCAGCACTTCACTCTG TTAAAGCATAAACAGCA-3′
5′-Y66H1B.4	5′-GAGGAATTCATGGATTCGGTTAAGCACACAACCG-3′
3′-Y66H1B.4	5′-AGCCTCGAGTTAATTAGAAGTT GAAGGTGGAGC-3′
5′-T07A9.1	5′-GAGAATTCATGGAATCGATATCACGTTCG-3′
3′-T07A9.1	5′-AGGGGCTCGAGTTAAAATGATTTTAAAAAATTG-3′
*Pst*IY66H1B.4	5′-TTTACTGCAGCATTTCTACTCCAC-3′
*Sma*IY66H1B.4	5′-CGAATCCCGGGTAACCTGAAAA-3′
*Pst*IT07A9.1	5′-TTAATCTGCAGAAAGCAATGGGAATCGTGAGAATC-3′
*Sma*IT07A9.1	5′-TCGTTCCCGGGTATTCGGACAATCAGGTGCTCCAGC-3′

In this study, we have identified the gene encoding *Caenorhabditis elegans* SPL using nucleotide sequence homology and complementation of a yeast SPL mutant. SPL promoter analysis using green fluorescent protein (GFP) reporter constructs indicates tissue specific and developmental regulation of the SPL gene. RNA interference (RNAi) was used to investigate the importance of SPL in regulating metabolism of sphingolipid signaling molecules and the role of SPL *in vivo* during development and in the adult worm. Our results demonstrate that *C. elegans* SPL expression is localized to intestinal cells and that interference with SPL expression leads to accumulation of phosphorylated and unphosphorylated long-chain bases. Furthermore, inhibiting SPL expression results in severe intestinal damage, egg laying defects, and semi-lethality. These studies demonstrate the importance of SPL in maintaining tight regulation of bioactive sphingolipid metabolites and indicate that SPL expression has a role in normal gut and reproductive physiology, representing a first step toward understanding the biological importance of the sphingolipid degradative pathway in more complex animals, including humans.

EXPERIMENTAL PROCEDURES

Cloning of C. elegans SPL—Two open reading frames (gi1226312 = *B0222.4*; gi3801005 = *Y66H1B.4*) with significant homology to the *S. cerevisae* SPL gene were identified in the *C. elegans* genome by BLAST search of the GenBank™ data base (17). Both these open reading frames were reverse-transcribed and amplified by PCR from total *C. elegans* RNA using the Access RT-PCR system (Promega) and the DNA primer pairs 5′-B0222.4 and 3′-B0222.4, and 5′-Y66H1B.4 and 3′-Y66H1B.4 (Table I).

After restriction enzyme digestion, the resulting cDNA fragments were cloned into *Eco*RI (*B0222.4*) and *Eco*RI and *Xho*I (*Y66H1B.4*) sites of the pYES2 yeast shuttle vector (Invitrogen). The resulting galactose-inducible constructs pYES2-B0222.4 and pYES2-Y66H1B.4 were evaluated for ability to functionally and biochemically complement relevant *S. cerevisiae* mutant strains. For injected RNAi studies in *C. elegans*, the *B0222.4* and *Y66H1B.4* sequences were cloned into pBluescript at the *Eco*RI and *Eco*RI/*Xho*I sites, respectively. Additionally, cDNA of the *T07A9.1* gene, which is immediately upstream of *Y66H1B.4* on chromosome IV, was amplified by RT-PCR using the DNA primer pair 5′-T07A9 and 3′-T07A9, and the resulting DNA fragment was cloned into pBluescript (+ or −) at *Eco*RI and *Xho*I sites. RNA was produced from the resulting plasmids pBS-B0222.4, pBS-Y66H1B.4, and pBS-cT07A9.1 as described below.

Generation and Expression of C. elegans Reporter Gene Constructs—For transcriptional reporter constructs, a promoter region of 512 nucleotides of *Y66H1B.4* upstream untranslated sequence corresponding to a position −3 to −515 relative to the ATG start codon was generated by PCR from T07A9 cosmid template DNA using Vent DNA Polymerase (New England BioLabs) and the primer pair *Pst*IY66H1B and *Sma*IY66H1B. Similarly, a promoter region of 3949 nucleotides of *T07A9.1* upstream untranslated sequence corresponding to a position −2 to −3951 relative to the ATG start codon was generated by PCR from T07A9 cosmid template DNA using the primer pair *Pst*IT07A9 and *Sma*IT07A9. Following restriction enzyme digestion, these PCR products were cloned into *Pst*I and *Sma*I sites of the GFP reporter plasmids pPD95.75 and pPD95.67 (gifts of A. Fire and B. Meyer). The resulting constructs, pY66H1B.4.75, pY66H1B.4.67, pT07A9.1.75, and pT07A9.1.67, were used in transgenic experiments.

Creation of Transgenic Animals—Transformation of *C. elegans dpy-20(e1362)* worms with reporter constructs was performed by microinjection of plasmid DNA into the distal arm of the hermaphrodite gonad as described previously (26, 27). Reporter construct DNA was co-injected with plasmid pMH86, allowing the recognition of transformants by rescue of the Dpy phenotype (28). Two independently derived stable lines for each construct were analyzed, PS4246 *dpy-20(e1362); syEx561[pMH86,pY66H1B.4.75]* and PS4247 *dpy-20(e1362); syEx562[pMH86,pY66H1B.4.67]*. GFP was visualized by mounting live transgenic nematodes on 5% agarose 0.01% sodium azide and viewed by fluorescence microscopy (Zeiss Axioskop) using a Chroma High Q GFP long pass filter set (450-nm excitation and 505-nm emission).

Yeast Strains and Growth Conditions—Strain JSK133, a derivative of JK9–3d containing a *dpl1*::*TRP1* allele, was transformed with pYES2 vector control, pYES2-*DPL1*, pYES2-B0222.4, and pYES2-Y66H1B.4 using the lithium acetate method (see Table II) (29). Transformants were isolated by uracil prototrophy. Cells were grown in uracil media containing 20 g/liter of either glucose or galactose. Sphingosine resistance was analyzed using a dilutional assay, as previously reported (17). Cells were also plated onto standard YEPD (yeast extract, peptone, dextrose) media as a control. Sphingosine-enriched plates were made with minimal media containing 0.0015% Nonidet P-40 and 50 μM D-*erythro*-sphingosine. At this concentration of Nonidet P-40, no effects on cell viability are observed.

SPL Assay—Yeast strains of interest were grown to mid-logarithmic phase (A_{600} = 1.0) in uracil media containing galactose as a carbon source. Whole cell extracts were prepared as described previously (17), adjusted for protein concentration, and evaluated for SPL activity as described using as substrate [^{3}H]dihydrosphingosine 1-phosphate, obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) (30). Similarly, worms propagated on *Escherichia coli* HB101 strain carrying either no vector or a *Y66H1B.4* RNAi feeding construct were harvested, washed in M9 and SPL extraction buffer, homogenized using a Wheaton glass homogenizer, adjusted for protein concentration and evaluated for SPL activity as above. Extracts containing only denatured proteins were prepared by boiling for 5 min.

Worm Propagation and Collection of Staged Animals—Worms were grown under standard conditions in nematode growth medium (31). To synchronize *C. elegans* cultures worms were grown in liquid culture, treated with alkaline hypochlorite treatment, resuspended in S basal media, and harvested at various time points to obtain L4 larvae for feeding experiments described below.

RNA Interference by Injection—The *B0222.4*, *Y66H1B.4*, and *T07A9.1* cDNAs were cloned into pBluescript in the multiple cloning site. RNA complementary to each strand was synthesized using T3 and T7 promoter regions and an *in vitro* transcription kit (Promega). The two strands were annealed to make double-stranded RNA (dsRNA) and injected into the distal gonads of 12 wild-type (N2 Bristol) young adult *C. elegans* hermaphrodites as described (27). Uninjected hermaphrodites and hermaphrodites injected with a control dsRNA were handled in parallel as controls. Eight hours after injection, each hermaphrodite was transferred to a fresh culture plate, and 12-h cohorts of F1 progeny were established. Progeny were examined daily with a dissecting microscope and by Nomarski microscopy (Zeiss Axioscop) until most animals reached adulthood and culture plates became too crowded with F2 progeny (32).

RNA Interference by Ingestion—Wild-type (N2 Bristol) L4 larvae were plated on *E. coli* strain HB101 carrying an L440 plasmid containing the *Y66H1B.4* insert (kind gift of Julie Ahringer, Cambridge, UK) (33, 34). This plasmid expresses the corresponding target gene dsRNA when induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

TABLE II
Yeast strains used in these experiments

Name	Relevant genotype
JSK133	**a** *dp1*::*Trp*
JS200	**a** *dp11*::*Trp* [*pYES2-B0222.4 (URA3)*]
JS201	**a** *dp11*::*Trp* [*pYES2-Y66H1B.4 (URA3)*]
JS203	**a** *dp11*::*Trp* [*pYES2-DPL1 (URA3)*]

L4 worms were fed and allowed to lay eggs, as described previously (35). Progeny were harvested and evaluated by microscopy and biochemical analysis, as described above.

Labeling of C. elegans Lipids—Worms were treated by RNAi by ingestion as described above, except that worms were plated on IPTG plates at the L1 stage and were harvested at L4 to adult stage. Worms were then collected by scraping and resuspended in 1 ml of S basal media containing 50 μM [^{3}H]serine (100 μCi/ml) and incubated for 4 h at 20 °C. Following incubation the volume was brought up to 50 ml with M9 buffer, and worms were pelleted by centrifugation at 3000 $\times$ *g* for 10 min at 4 °C. Supernatant was discarded, and worms were washed two times with 50 ml of M9 buffer each time. Washed worms were frozen in liquid nitrogen and lyophilized until a flaky powder was obtained. Lyophilized worms were stored at −80 °C until used.

Preparation and Analysis of C. elegans Lipid Extracts—A sample containing 15 mg of lyophilized worms was placed in a glass tube, and 2 ml of ice-cold chloroform/methanol, 1:1 (v/v), was added to the tube. The sample was homogenized by tip sonication (5 $\times$ 20 s) while on ice and then centrifuged at 1500 $\times$ *g* for 10 min. Supernatant was recovered, and a second extraction was performed on the pellet. The two supernatants were pooled and dried down in a Speed Vac. Lipid extract was resuspended in 400 μl of 1.5 M ammonium hydroxide by tip sonication, and 2 ml of chloroform/methanol, 2:1 (v/v), was added while vortexing. The sample was centrifuged at 1500 $\times$ *g* for 10 min to obtain a clear separation of the two phases. Both phases were recovered and dried down in a Speed Vac. To compare the efficiency of the homogenization and extraction procedure between different samples, the total amount of phosphatidylcholine present in the organic phase was determined following phospholipase B treatment (Waco Chemicals, Neuss, Germany). Lipid equivalent to 250 μg of phosphatidylcholine was removed for TLC analysis. Lipid groups were separated by running TLC (Silica Gel HL, 20 $\times$ 20 cm, Analtech, Newark, DE) and identified based on known standards. Different solvent systems were applied. Phospholipids were analyzed by applying 10–20% of the organic phase to a TLC plate followed by a separation using the solvent system chloroform/methanol/glacial acidic acid/0.9% sodium chloride, 20:10:3.2:1 (v/v, solvent system A). The remainder of the organic phase was subjected to mild alkaline hydrolysis by incubating for 1 h at 37 °C in 400 μl of methanol containing 0.1 M potassium hydroxide. Following hydrolysis the lipids were dried down, resuspended in chloroform/methanol, 2:1 (v/v), and applied to a TLC plate. Lipids were separated using two-dimensional TLC. Plates were developed in the first dimension using the solvent system chloroform/methanol/4.2 M ammonium hydroxide, 9:7:2 (v/v, solvent system B) followed by a separation in the second dimension using solvent system A. Spots corresponding to sphingoid bases and ceramide were identified. Phosphorylated sphingoid bases obtained in the water phase were analyzed using the same two-dimensional TLC procedure. Aliquots were taken out throughout the homogenization and extraction procedures and evaluated for radioactivity by scintillation counting. Spots corresponding to lipids of interest were scraped from the TLC plates and counted for radioactivity.

RESULTS

Identification of C. elegans SPL—Based on a BLASTP search of the GenBank™ data base against the *DPL1* (dihydrosphingosine phosphate lyase) gene sequence of *S. cerevisiae*, two open reading frames were identified in the *C. elegans* genome. Open reading frame *B0222.4* on cosmid B0222 (representing a portion of chromosome V) had been predicted to encode a glutamate decarboxylase. Predicted coding sequence *Y66H1B.4* was identified on yeast artificial chromosome Y66H1B (representing a portion of chromosome IV) and is noted to contain a conserved pyridoxal phosphate-dependent decarboxylase domain. No experimental evidence for the function of either gene was available. These two sequences are 39% identical and 60% similar to one another. *Y66H1B.4* is slightly more similar to the SPL protein sequences of *S. cerevisiae* and *Homo sapiens* (40% identical to *DPL1* and 41% identical to human SPL) compared with *B0222.4* (36% identical to *DPL1* and 38% identical to human SPL). In each case, the region of similarity spans nearly the entire length of each protein sequence. A predicted pyridoxal 5′-phosphate binding lysine (shown in Fig. 1) is present in both *B0222.4* and *Y66H1B.4*. However, eliminating the conserved region surrounding this lysine from the comparison did not significantly affect the overall sequence similarity. To determine whether *B0222.4* and/or *Y66H1B.4* encode a functional SPL, cDNA from each transcript was created by reverse transcription from total *C. elegans* RNA, amplified by PCR, and cloned into the pYES2 yeast shuttle vector, in which genes are induced in the presence of galactose. Both constructs were then transformed into a haploid *S. cerevisiae* strain containing a null *dpl1* allele, the sole endogenous SPL gene of this organism. This strain is extremely sensitive to the presence of D-*erythro*-sphingosine in the media due to an inability to catabolize this lipid. When active SPL proteins are expressed in this background, sphingosine sensitivity is abrogated. As shown in Fig. 2*A*, *Y66H1B.4* expression in media containing galactose afforded substantial growth of the *dpl1* strain on sphingosine, whereas *B0222.4* expression supported only marginal growth (Fig. 2*B*). To determine whether *B0222.4* and/or *Y66H1B.4* were capable of restoring SPL activity to the *dpl1* mutant, whole cell extracts of *dpl1* strains expressing either *Y66H1B.4* or *B0222.4* were prepared and evaluated for activity against a radioactive dihydrosphingosine 1-phosphate substrate. Although *Y66H1B.4* expression restored SPL activity to the *dpl1* strain, *B0222.4* did not (Fig. 3). These findings indicate that *Y66H1B.4* encodes the *C. elegans* SPL, and we have renamed this gene *spl-1*.

C. elegans SPL Is Expressed in a Tissue-specific Manner—The organization of the *spl-1* gene is shown in Fig. 4. The coding region of *spl-1* resides within a 3.2-kb sequence containing 10 exons and 9 introns on chromosome IV. The intragenic region between *spl-1* and its nearest 5′ neighbor (hypothetical gene *T07A9.1*) is 521 nucleotides. Due to the small size of this region, Wormbase (available at www.wormbase.org) predicts that *spl-1* may be a downstream gene in an operon with *T07A9.1*. Therefore, to evaluate gene expression from the *spl-1* locus, GFP reporter constructs were created from both potential regulatory regions, which are located immediately 5′ to *spl-1* and to the large intragenic region upstream of *T07A9.1*. Reporter constructs with and without nuclear localization sequences were created to aid in interpretation of the fluorescent signal. Reporter constructs and *dpy-20+* plasmid pMH86 were simultaneously microinjected into the distal arm of the hermaphrodite gonad of *dpy-20(e1362)* worms. Transformants were identified by rescue of the Dpy phenotype (28). Lines in which the F2 and subsequent generations demonstrated the rescue phenotype were considered stable and were analyzed for GFP expression. As shown in Fig. 5, GFP expression under regulation of the *spl-1* immediate upstream region was pronounced and localized almost exclusively in the intestine. All cells of the intestine expressed *spl-1*. In line PS4246, which transmits the non-Dpy phenotype at 98%, most animals demonstrated a fluorescent signal in the region just posterior to the pharynx with no expression in the rest of the anterior gut. Strong expression was also observed in the posterior gut of most animals. No expression was detected in the gonad, body-wall muscle, pharynx, vulva, or epidermis. Although a low percentage of animals showed some expression in a pair of cells in the head, expression was too weak and variable to identify these cells. This expression pattern was consistent in worms transformed with nuclear localizing reporter constructs and

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H. sapiens      ----------MPSTDLLMLKAFEPYLEILEVYSTKAKNYVNGHCT--------KYEP     39
M. musculus     ----------MPGTDLLKLKDFEPYLEILESYSTKAKNYVNGYCT--------KYEP     39
B0222.4         ----------------------------MDFALEQYHSAKDLLIFELR--------KFNP     24
Y66H1B.4        ----------------------MDSVKHTTEIIVDLTKMHYHMINDRLS--------RYDP     31
S. cerevisiae   MSGVSNKTVSINGWYGMPIHLLREEGDFAQFMILTINELKIAIHGYLRNTPWYNMLKDYL     60
                             .          ::         :          :
H. sapiens      WQLIAWSVVWTLLIVWGYEFVFQPES-LWSRFKKKCFKLTRKMPIIGRKIQDKLNKTKDD     98
M. musculus     WQLIAWSVLCTLLIVWVYELIFQPES-LWSRFKKKLFKLIRKMPFIGRKIEQQVSKAKKD     98
B0222.4         IVLVSSTIVATYVLTNLRHMHLDEMG-IRKRLSTWFFTTVKRVPFIRKMIDKQLNEVKDE     83
Y66H1B.4        VVLVLAAFGGTLVYTKVVHLYRKSEDPILKRMGAYVFSLLRKLPAVRDKIEKELAAEKPK     91
S. cerevisiae   FVIFCYKLISNFFYLLKVYGPVRLAVRTYEHSSRRLFRWLLDSPFLRGTVEKEVTKVKQS    120
                 :.  .  .  .          .:     *      * :   ::.::   * .
H. sapiens      ISKNMSFLKVDKEYVKALPSQGLSSSAVLEKLKEYSSMDAF--WQEGRASGTVYS--GEE    154
M. musculus     LVKNMPFLKVDKDYVKTLPAQGMGTAEVLERLKEYSSMDGS--WQEGKASGAVYN--GEP    154
B0222.4         LEKSLRIVDRSTEYFTTIPSHSVGRTEVLRLAAIYDDLEGPA-FLEGRVSGAVFNREDDK    142
Y66H1B.4        LIESIHKDDKDKQFISTLPIAPLSQDSIMELAKKYEDYNTFN-IDGGRVSGAVYT-DRHA    149
S. cerevisiae   IEDELIRSDSQLMNFPQLPSNGIPQDDVIEELNKLNDLIPHTQWKEGKVSGAVYH--GGD    178
                : ..:   . .   .  :*   :    ::.     ..         *:.**:*:
H. sapiens      KLTELLVKAYGDFAWSNPLHPDIFPGLRKIEAEIVRIACSLFNGGPDS-CGCVTSGGTES    213
M. musculus     KLTELLVQAYGEFTWSNPLHPDIFPGLRKLEAEIVRMTCSLFNGGPDS-CGCVTSGGTES    213
B0222.4         DEREMYEEVFGKFAWTNPLWPKLFPGVRIMEAEVVRMCCNMMNGDSET-CGTMSTGGSIS    201
Y66H1B.4        EHINLLGKIYEKYAFSNPLHPDVFPGARKMEAELIRMVLNLYNGPEDS-SGSVTSGGTES    208
S. cerevisiae   DLIHLQTIAYEKYCVANQLHPDVFPAVRKMESEVVSMVLRMFNAPSDTGCGTTTSGGTES    238
                .  .:    : .:  :* * *.:**. * :*:*:: :   : *.  :: .*  ::**: *
H. sapiens      ILMACKAYRDLAFE-KGIKTPEIVAPQSAHAAFNKAASYFGMKIVRVPLT-KMMEVDVRA    271
M. musculus     ILMACKAYRDLALE-KGIKTPEIVAPESAHAAFDKAAHYFGMKIVRVALK-KNMEVDVQA    271
B0222.4         ILLACLAHRNRLLK-RGEKYTEMIVPSSVHAAFFKAAECFRIKVRKIPVDPVTFKVDLVK    260
Y66H1B.4        IIMACFSYRNRAHS-LGIEHPVILACKTAHAAFDKAAHLCGMRLRHVPVD-SDNRVDLKE    266
S. cerevisiae   LLLACLSAKMYALHHRGITEPEIIAPVTAHAGFDKAAYYFGMKLRHVELDPTTYQVDLGK    298
                :::** : :       *   . ::.  :.**.* ***    ::: :: :    .**:
H. sapiens      MRRAISRNTAMLVCSTPQFPHGVIDPVPEVAKLAVKYKIPLHVDACLGGFLIVFMEKAGY    331
M. musculus     MKRAISRNTAMLVCSTPQFPHGVMDPVPEVAKLTVRYKIPLHVDACLGGFLIVFMEKAGY    331
B0222.4         MKAAINKRTCMLVGSAPNFPFGTVDDIEAIGQLGLEYDIPVHVDACLGGFLLPFLEED--    318
Y66H1B.4        MERLIDSNVCMLVGSAPNFPSGTIDPIPEIAKLGKKYGIPVHVDACLGGFMIPFMNDAGY    326
S. cerevisiae   VKKFINKNTILLVGSAPNFPHGIADDIEGLGKIAQKYKLPLHVDSCLGSFIVSFMEKAGY    358
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H. sapiens      PLEHPFDFRVKGVTSISADTHKYGYAPKGSSLVLYSDKKYRNYQFFVDTDWQGGIYASPT    391
M. musculus     PLEKPFDFRVKGVTSISADTHKYGYAPKGSSVVMYSNEKYRTYQFFVGADWQGGVYASPS    391
B0222.4         --EIRYDFRVPGVSSISADSHKYGLAPKGSSVVLYRNKELLHNQYFCDADWQGGIYASAT    376
Y66H1B.4        -LIPVFDFRNPGVTSISCDTHKYGCTPKGSSIVMYRSKELHHFQYFSVADWCGGIYATPT    385
S. cerevisiae   KNLPLLDFRVPGVTSISCDTHKYGFAPKGSSVIMYRNSDLRMHQYYVNPAWTGGLYGSPT    418
                      ***  **:***.*:**** :*****:::* ...    *::  . * **:*.:.:
H. sapiens      IAGSRPGGISAACWAALMHFGENGYVEATKQIIKTARFLKSELEN-IKGIFVFGNPQLSV    450
M. musculus     IAGSRPGGIIAACWAALMHFGENGYVEATKQIIKTARFLKSELEN-IKNIFIFGDPQLSV    450
B0222.4         MEGSRAGHNIALCWAAMLYHAQEGYKANARKIVDTTRKIRNGLSN-IKGIKLQGPSDVCI    435
Y66H1B.4        IAGSRAGANTAVAWATLLSFGRDEYVRRCAQIVKHTRMLAEKIEK-IKWIKPYGKSDVSL    444
S. cerevisiae   LAGSRPGAIVVGCWATMVNMGENGYIESCQEIVGAAMKFKKYIQENIPDLNIMGNPRYSV    478
                : ***.*   . .**:::  ..: *     :*:  :  : . :.: *  :   * .  .:
H. sapiens      IALGSR-DFDIYRLSNLMTAKGWNLNQLQFPPSIHFCITLLHARKRVAIQFLKDIRESVT    509
M. musculus     IALGSN-DFDIYRLSNMMSAKGWNFNYLQFPRSIHFCITLVHTRKRVAIQFLKDIRESVT    509
B0222.4         VSWTTNDGVELYRFHNFMKEKHWQLNGLQFPAGVHIMVTMNHTHPGLAEAFVADCRAAVE    495
Y66H1B.4        VAFSGN-GVNIYEVSDKMMKLGWNLNTLQNPAAIHICLTINQANEEVVNAFAVDLEKICE    503
S. cerevisiae   ISFSSK-TLNIHELSDRLSKKGWHFNALQKPVALHMAFTRLSAH--VVDEICDILRTTVQ    535
                ::   .  .:::.. : :    *::* ** * .:*: .*   :.  :.  :    .
H. sapiens      QIMKN--PKAKTTGMGAIYGMAQTTVDRNMVAELSSVFLDSLYSTDTVTQGSQMNGSPKP    567
M. musculus     QIMKN--PKAKTTGMGAIYGMAQATIDRKLVAEISSVFLDCLYTTDPVTQGNQMNGSPKP    567
B0222.4         FVKSHKPSESDKTSEAAIYGLAQSIPDRSLVHEFAHSYIDAVYALTE-------------    542
Y66H1B.4        ELAAKGEQKAD-SGMAAMYGMAAQVP-KSVVDEVIALYIDATYSAPPSTSN---------    552
S. cerevisiae   ELKSESNSKPSPDGTSALYGVAGSVKTAGVADKLIVGFLDALYKLGPGEDTATK------    589
                 :  .   :..  . .*:**:*       :. :.   ::*. *      .    ..:...
H. sapiens      H 568
M. musculus     R 568
```

FIG. 1. **Multiple sequence alignment.** Sequences were aligned using the ClustalW program (55). Conserved pyridoxal 5′-phosphate binding lysine is shown in *boldface*.

non-nuclear constructs. In contrast, only weak and non-tissue-specific expression from the *T07A9.1* promoter constructs was observed (data not shown).

C. elegans SPL Expression Is Developmentally Regulated—To determine whether *spl-1* expression is developmentally regulated, GFP fluorescence was analyzed in transgenic animals at all developmental stages. Although we were not able to distinguish non-Dpy nematodes during embryogenesis, early to comma stage embryos in line PS4246, which shows 98% transmission of the transgene in larvae and adults, were devoid of expression and expression in pretzel stage embryos was barely detectable (Table III). In both transgenic lines analyzed, the majority of L1 larvae and 100% of L2, L3, and L4 larvae and adults showed medium to high expression, with the highest levels of expression noted in older adults (Table III).

SPL Expression Is Required for Normal Development in C. elegans—To assess whether the sphingolipid degradative pathway serves a role in *C. elegans* development, *spl-1* expression was inhibited using RNA interference. This method provides a potent and specific block to protein expression by inducing degradation of the targeted endogenous mRNA. Toward that end, full-length SPL dsRNA was created by *in vitro* transcription and injected into adult hermaphrodite worms, as described under "Experimental Procedures." Animals injected with full-length *B0222.4* dsRNA and uninjected animals were employed as negative controls. The resulting F1 progeny were analyzed throughout development until they reached adulthood.

Results representative of 12 separate injections of SPL dsRNA are shown in Fig. 6. For SPL dsRNA-injected worms, all eggs laid after ~12 h post-injection demonstrated abnormalities, although these ranged broadly in severity from worms that did not develop beyond L1 or L2 stages to worms that developed to adulthood and demonstrated defects of adult structures. Compared with control F1s, animals inheriting *spl-1* dsRNA developed slowly, moved sluggishly, and were thin, pale, and starved in appearance. The adults were smaller than controls (Fig. 6*A*) and did not pump food actively. The treated animals reached adulthood 24–48 h later than controls. Adult hermaphrodites that inherited *spl-1* dsRNA were markedly different from controls especially in the gonad and uterus. Although control animals had abundant nuclei in the distal gonad and a row of developing oocytes in the proximal gonad, affected hermaphrodites had poorly developed distal gonads with fewer nuclei (Fig. 6*B*). Control adults had embryos of progressive stages of development in the uterus, whereas the number of developing oocytes in the proximal gonad of affected hermaphrodites was significantly reduced. The embryos in the uterus of affected progeny were also morphologically abnormal. Those near the vulva were at late developmental stages, indi-

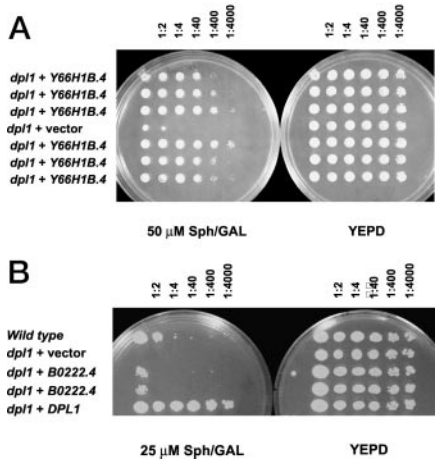

FIG. 2. **Complementation of a yeast SPL mutant.** *A*, functional complementation by *Y66H1B.4* of yeast strain JSK133, which contains a null allele of the endogenous yeast SPL, *DPL1*. Saturated cultures of JSK133 transformed with pYES2 vector (*dpl1 + vector*) or with pYES2-Y66H1B.4 (*dpl1+ Y66H1B.4*) were adjusted for $A_{600} = 5$ and serially diluted as indicated. Cells were template-inoculated onto YEPD plates or plates containing sphingosine and galactose as a carbon source, as described under "Experimental Procedures." *B*, same as above, except that strains include wild type, JSK133 plus vector, JSK133 plus pYES2-B0222.4 (*dpl1 + B0222.4*), and JSK133 plus pYES2-DPL1 (*dpl1 + DPL1*). This is representative of three separate experiments.

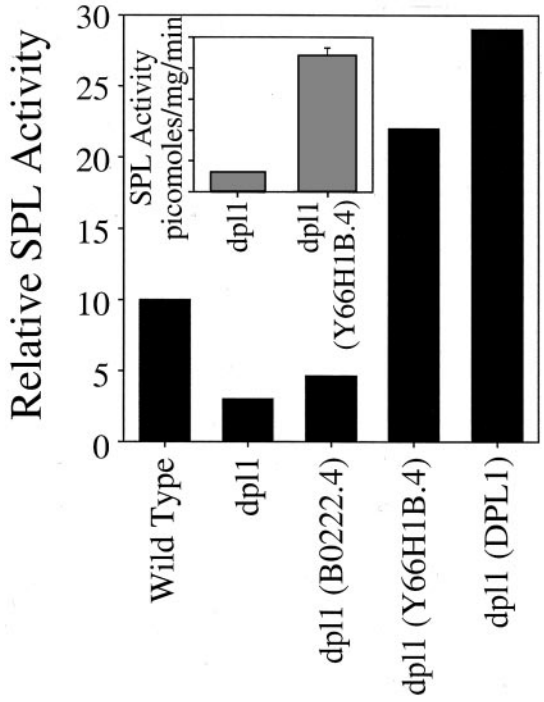

FIG. 3. **SPL activity of *Y66H1B.4* expressed in yeast.** Whole cell extracts of yeast strains were evaluated for SPL activity. Relative and absolute SPL activities are shown for *Y66H1B.4*. For *dpl1 versus dpl1* (*Y66H1B.4*), $p < 0.003$. This is representative of two separate experiments.

cating a defect in egg laying. There was not a uniform progression of developmental stages in adjacent embryos suggesting a defect in ovulation or cell division (Fig. 6, *C–E*). Embryonic and larval semi-lethality was observed, and some premature death of adults was also noted. None of these effects were found in worms that inherited *B0222.4* dsRNA, which were similar in growth, appearance, and reproduction to uninjected worms. Furthermore, no synergistic effects were observed when *B0222.4* and *spl-1* dsRNA were injected together.

The Product of the spl-1 Gene Is the Predominant SPL of C. elegans—It has been shown previously that RNAi can be performed by feeding *C. elegans* bacteria containing plasmid constructs that express dsRNA of interest (34). The advantages of this method include its technical simplicity, the large numbers of affected progeny allowing biochemical analysis of treated populations, and ability to manipulate the potency of the effect by altering induction conditions, facilitating the identification of hypomorphic phenotypes. To confirm the developmental phenotypes observed in RNAi by injection, to seek additional phenotypes, and to determine the effect on SPL activity, feeding RNAi was undertaken. Comparison of control worms *versus* worms treated with *spl-1* RNAi by ingestion indicates that SPL enzyme activity is completely inhibited by this procedure (Fig. 7). Therefore, the enzyme encoded by *spl-1* is responsible for most, if not all, SPL activity in the worm.

Inhibition of C. elegans SPL Expression Leads to Intestinal Cell Death and Egg Laying Defects—Using feeding RNAi, phenotypes similar to those observed in injection RNAi were appreciated, including pallor, shrunken gonads, bloated appearance, and unlaid eggs (Fig. 8). Rare adult hermaphrodite worms were identified with hatched larvae within them (the "bag of worms" phenotype). Pallor was a consistent finding, present in all progeny of treated animals. Furthermore, the intestines of many worms appeared strikingly abnormal, shrunken and vacuolated. The onset of these defects was late. Worms appeared normal throughout the larval stages, whereas pallor became evident as worms reached early adulthood, and more pronounced phenotypes were appreciated only in adults more than 24 h old. These phenotypes persisted in adult worms transferred to non-transformed bacterial plates, indicating that the defects caused by SPL inhibition are irreversible. Whereas egg laying was unaffected in worms fed RNAi as adults, it was modestly diminished in worms subjected to RNAi throughout development (data not shown).

The Observed Developmental Defects Are Specific to spl-1—Although the reporter studies described above indicate that *spl-1* is transcribed independently of *T07A9.1* and is unlikely to be in an operon regulated by the *T07A9.1* promoter, it was important to verify that the phenotypes observed in *spl-1* RNAi-treated worms were caused by inhibition of *spl-1* expression rather than a polar effect leading to a block in expression of the hypothetical *T07A9.1* protein product. To maximize the potency of the block in expression, injected RNAi was employed to inhibit expression of *T07A9.1*. Importantly, inhibition of *T07A9.1* expression resulted in no demonstrable effects on egg laying, gonadal development, or growth of the injected worms (data not shown). Therefore, it appears that specific inhibition of *spl-1* expression produces the aforementioned abnormalities in the growth, developmental progression, and reproduction of *C. elegans*.

Inhibition of C. elegans SPL Expression Leads to Accumulation of Phosphorylated and Unphosphorylated Long-chain Bases—To gain insight into potential mechanisms responsible for the developmental defects observed in SPL-deficient worms, the effect of *spl-1* RNAi on serine incorporation into worm sphingolipids and phospholipids was examined, as described under "Experimental Procedures." As shown in Table IV, *spl-1* RNAi treatment was associated with a 1.7-fold increase in the amount of serine incorporated into phosphorylated long-chain bases and a 2.7-fold increase in unphosphorylated long-chain bases in comparison to control worms. In contrast, serine incorporation into ceramide, phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine was not appreciably altered in the RNAi worms. Thus, specific accumulation of phosphorylated and unphosphorylated long-chain bases results

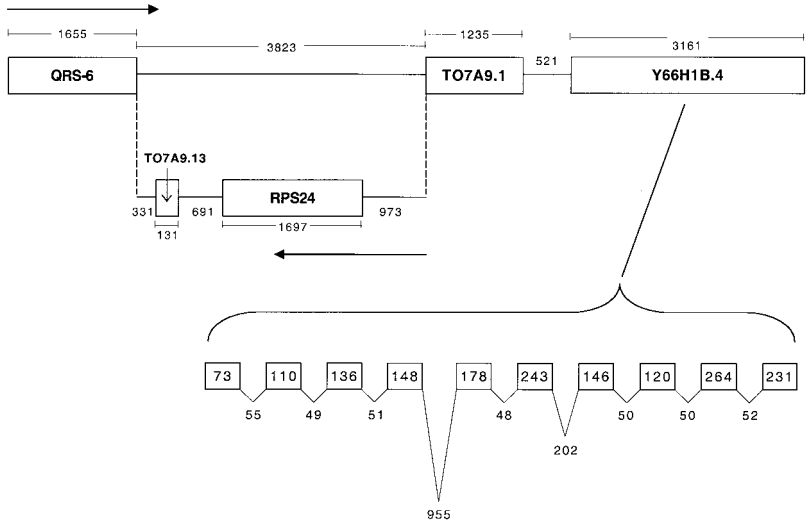

FIG. 4. **Organization of the *C. elegans* SPL gene.** Shown are *Y66H1B.4* and its gene organization, *T07A9.1*, and the two potential 5′ regulatory regions for *Y66H1B.4* (*T07A9.1-Y66H1B.4* intragenic region and *QRS-6-T07A9.1* intragenic region). *RPS24* is encoded by the opposite DNA strand, as indicated by the *arrows*.

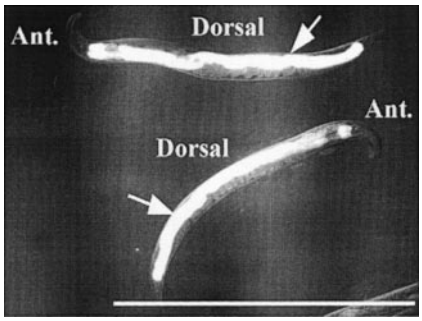

FIG. 5. ***C. elegans* SPL expression using GFP reporter transgenes.** Two adult hermaphrodites of strain PS4246 showing GFP expression throughout the intestine (*arrows*) are shown. *Scale bar* represents 1 mm. Nomarski and fluorescent images were merged using PhotoShop. *Ant.*, anterior.

TABLE III
GFP reporter expression during development

Developmental stage	Number scored	% Expressing GFP
		%
Pre-comma stage embryos	24	0
Comma stage embryos	9	0
Pretzel stage embryos	22	23
L1 larvae	22	73
L2 larvae	20	100
L3 larvae	20	100
L4 larvae	21	100
Adults	25	100

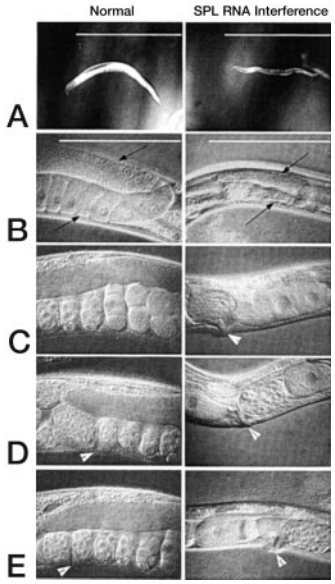

FIG. 6. **Worms treated with *spl-1* RNAi by injection.** Nomarski images of adult hermaphrodites of both untreated (*left panels*) and treated (*right panels*) nematodes are shown. In *all panels*, dorsal orientation is *upward*. *A*, images of the entire hermaphrodite showing the difference in body size between treated and untreated individuals. Anterior is to the *left*. *Scale bar*, 1 mm. *B*, section of the proximal (*lower arrows*) and distal (*upper arrows*) gonad arms showing reduced size and structural abnormalities in the treated hermaphrodite. *C–E*, the uterus in the region of the vulva (*arrowheads*) showing a progression of embryonic cell division from *right* to *left* in the untreated animals and the presence of both advanced (multicellular) and delayed/abnormal embryos (one-two nuclei) in the treated hermaphrodites. The vulva in the normal hermaphrodite in *panel C* is just to the *left* of the field of view. The *scale bar* in *panel B* represents 0.1 mm and applies to *B–E*.

from RNAi treatment and may contribute to the reproductive and anatomical defects observed in *spl-1*-deficient worms.

DISCUSSION

In this study, we have identified the *C. elegans* SPL gene as "*spl-1*" using nucleotide sequence homology searches and functional complementation in yeast. This gene appears to encode the sole or predominant SPL in *C. elegans*, because inhibition of its expression is associated with undetectable levels of SPL activity *in vivo*.

C. elegans spl-1 expression is regulated in a tissue-specific manner, with robust expression detected in intestinal cells and faint expression in the head. Our data are in agreement with a more extensive *C. elegans* developmental analysis presented by the Nematode Expression Pattern Data base (36). This highly restricted tissue specificity is unlike that found in *Homo sapiens* and *Mus musculus*, where SPL is expressed in most tissues. Little is known about the physiology of *C. elegans* intestinal cells, although they have been shown to store nutrients and secrete digestive enzymes into the gut lumen and are likely to carry out detoxification, storage, and metabolic functions (37). Although in its natural environment *C. elegans* might ingest plant particles containing sphingolipids, laboratory strains are raised solely on *E. coli*, which does not synthesize or contain sphingolipids. Intermediates that accumulate in the absence of SPL activity may, thus, be derived from endogenous pathways or potentially from trace sphingolipids present in the culture media. It is possible that the localization of *spl-1* points toward

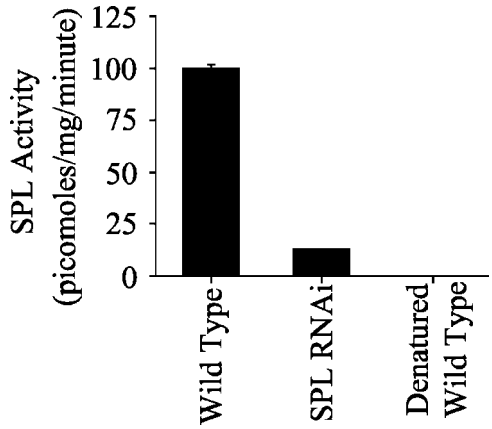

FIG. 7. **SPL activity after RNAi by ingestion.** Worms were treated with RNAi constructs as described in Fig. 8. Control animals were grown on plates containing IPTG and OP50 (no RNAi construct). At 48 h, worms were harvested, washed to remove bacteria, and homogenized using a Wheaton glass homogenizer followed by vortexing in the presence of glass beads. Extracts were normalized for protein content and assayed for SPL activity as described under "Experimental Procedures." For RNAi-treated *versus* untreated worms, $p < 0.001$. Residual SPL activity in RNAi-treated worms is apparent when compared with a denatured extract from wild type worms (*Denatured Wild Type*).

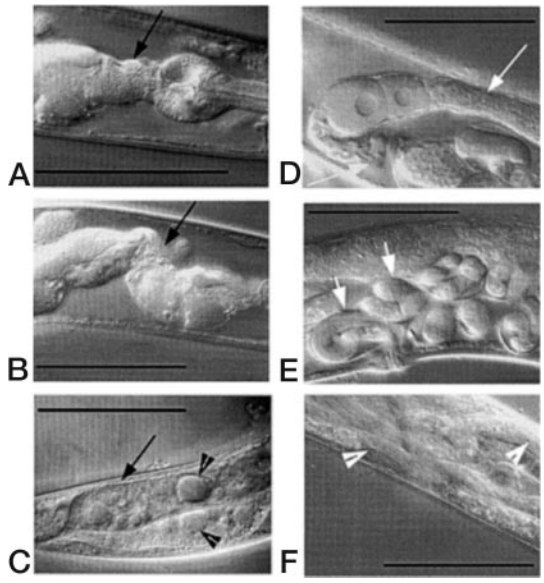

FIG. 8. **Worms treated with *spl-1* RNAi by ingestion.** Wild type N2 Bristol hermaphrodite worms were grown on plates containing bacteria expressing an *spl-1* RNAi construct. After 48 h, animals were evaluated in comparison to worms grown on plates containing IPTG and OP50, which does not contain an RNAi vector. Images are obtained using Nomarski optics, as described under "Experimental Procedures." This is representative of three separate experiments. In *all panels*, adult hermaphrodites are shown. Anterior is to the *right*; dorsal is *upward*. The *scale bar* shown in *A* represents 0.1 mm and applies to *A–F*. *A*, intestine (*black arrow*) is shrunken and appears constricted just posterior to the pharyngeal bulb. *B*, anterior portion of the intestine (*black arrow*) is shrunken and constricted. *C*, posterior portion of the intestine (*black arrow*) contains vacuoles (*arrowheads*). *D*, both proximal (*left white arrow*) and distal (*right white arrow*) regions of the gonad are shrunken. *E*, contents of the uterus include abnormally late stage embryos (*short white arrows*). *F*, a bag of worms evidenced by the presence of hatched embryos within the mother. *Arrowheads* indicate small larval pharyngeal bulbs.

a critical function in gut development and/or maintenance of intestinal integrity. Interestingly, we have observed a similar intestine-specific expression pattern in the SPL of *D. melanogaster*, indicating that this may be a widespread phenomenon in invertebrate organisms (38). Furthermore, the finding of *spl-1* expression in the head indicates a potential role in neurodevelopment, which is consistent with evidence suggesting a role for lysophospholipid signaling in nervous system development and function in mammals (39).

The onset of *spl-1* expression occurs at the time of egg hatching, and expression remains high throughout the life of the organism. The results of our GFP reporter assays indicate that the small intragenic region immediately upstream of *Y66H1B.4* is the true *spl-1* promoter. It appears that this ~500-bp region is sufficient to provide both spatial and temporal regulation of *spl-1* gene expression. Systematic study of this promoter using reporter gene constructs, mutational analysis, and deletional analysis should allow identification of the specific sequences involved in the regulation of *spl-1* expression. Analysis of sphingolipid metabolites throughout development, in combination with the use of transgenic approaches resulting in ectopic and/or constitutive *spl-1* expression, should elucidate the functional significance of this pattern of gene expression.

Importantly, *C. elegans spl-1* expression is critical for the maintenance of intestinal integrity and normal reproduction. Without *spl-1* expression, animals become bloated, pale, congested with eggs and hatched larvae, and demonstrate shrunken gut and gonadal structures. The finding of a phenotype involving intestinal cell integrity in SPL-inhibited animals is consistent with its observed expression pattern, whereas effects on the gonad are less directly explained. It could be postulated that the gonadal and egg laying defects observed in the *spl-1* RNAi-treated worms is due to a nonspecific effect of starvation. However, this is not likely, because this type of reproductive defect has not been described in other mutants in which starvation is a prominent feature (40). Furthermore, diminished egg laying was observed only in worms treated with *spl-1* RNAi throughout development, suggesting that this defect has a developmental basis. Interestingly, the intestine appears to play a critical role in the nurture of germ cells by producing yolk proteins that are imported into the developing oocytes and serving as nurse cells for the primordial germ cells (37). Therefore, potential toxicity to intestinal cells induced by S1P accumulation or other metabolic disturbances associated with *spl-1* inhibition may affect egg maturation by blocking this supportive role of intestinal cells in reproduction. S1P may have direct effects on reproduction, provided it can move from intestinal cells to other tissues through normal diffusion, secretion, or facilitated transport. This concept is supported by the observation that S1P concentration gradients regulate mammalian endothelial and smooth muscle cell migration through receptor signaling mechanisms (41, 42). Alternatively, once intestinal cell membranes are disrupted and the integrity of the intestinal tract has been violated, S1P and/or other metabolites and digestive enzymes may escape, causing pleiotropic effects on nearby organs.

Interestingly, the vacuolization, pallor, shrinkage, and ultimate destruction of the intestines, as well as the gonadal defects observed in worms treated with *spl-1* RNAi are remarkably similar to that observed in worms treated with Cry proteins of the *Bacillus thuringiensis* (*Bt*) bacterium. These potent natural insecticides have been utilized to create transgenic food plants resistant to a wide variety of insect pests (43, 44). Although their precise mode of action is not known, these toxins induce cell damage by intercalating into the cell membranes of insect midgut epithelial cells, causing pore formation (45, 46). *C. elegans* also develops intestinal damage in response to some Cry proteins (47, 48). How might *Bt* toxin and *spl-1* inhibition induce the same effect on nematode intestinal cells? S1P accumulation may result in a direct or indirect disruption of the membrane integrity of the intestinal cell. Lysophospho-

TABLE IV
Incorporation of serine into lipids of wild type and SPL RNAi-treated worms

Values are shown as a mean ± S.D. for three independent measurements. *Numbers in parentheses* represent -fold increase as compared to wild type. Numbers are normalized to total uptake of radioactivity. Average total uptake was 5,000,000 dpm.

	LCBP[a]	LCB	Ceramide	PS	PC	PE
			dpm			
WT	2525 ± 132 (1.0)	2284 ± 42 (1.0)	59,413 ± 2217 (1.0)	48,524 ± 3819 (1.0)	14,4307 ± 2404 (1.0)	503,685 ± 24,695 (1.0)
ΔSPL	4414 ± 151 (1.7)	6221 ± 392 (2.7)	60,588 ± 1930 (1.0)	53,503 ± 1510 (1.1)	185,328 ± 9590 (1.3)	542,140 ± 17,292 (1.1)

[a]LCBP = long chain phosphorylated base; LCB = long chain base; PS = phosphatidylserine; PE = phosphatidylethanolamine; WT = wild type; ΔSPL = *spl-1* RNAi.

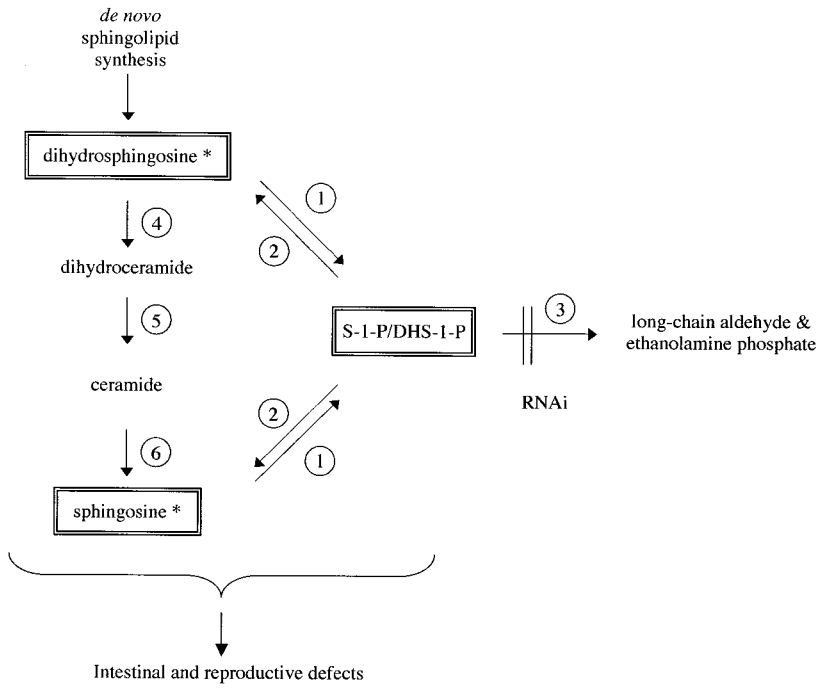

FIG. 9. **Sphingolipid accumulation in *spl-1* RNAi-treated worms.** *1*, sphingosine kinase; *2*, sphingosine phosphate phosphatase; *3*, sphingosine phosphate lyase (*spl-1*); *4*, ceramide synthase; *5*, sphingosine desaturase; *6*, ceramidase. Sphingolipid intermediates shown in *boxes* accumulate in *spl-1* RNAi-treated worms. *Asterisk*, note that sphingosine and dihydrosphingosine cannot be distinguished in the TLC assay system used in these experiments. Likewise, the phosphorylated forms cannot be distinguished.

lipids are known to exert various membrane modulating influences, including detergent effects that result in destabilization and disruption of membranes and chaperone-like effects, which facilitate protein folding (49–51). Either direct membrane disruption or interference with ion channels or other proteins required to maintain membrane stability and integrity could ultimately result in cell lysis. Other plausible mechanisms include inhibition of SPL-1 protein by *Bt* toxin, binding of *Bt* toxin to SPL-1 in membranes, or influences on downstream signaling events common to both the Bt toxin pathway and S1P pathways.

Finally, *spl-1* RNAi treatment led to significant accumulation of both phosphorylated and unphosphorylated long-chain bases, as would be expected from a block in SPL activity (Fig. 9). (In contrast, inhibiting expression of a putative *C. elegans* SK led to a substantial increase in long-chain bases but no increase in phosphorylated long-chain bases.)[2] The observed lipid changes are specific, in that phospholipid and ceramide levels are not significantly perturbed by loss of *spl-1* expression. Increases in long-chain bases of this magnitude are consistent with levels observed in mutant models lacking *spl-1* expression in other organisms, as well as in mammalian cells in which the SPL gene is disrupted and in mammalian cells overexpressing recombinant SK (38, 52–54). In each of the above cases, this degree of phosphorylated long-chain base accumulation was sufficient to induce signals and result in downstream biological effects. Thus, the perturbation of phosphorylated long-chain bases observed in our experiments could potentially exert significant physiological effects in the developing and adult worm.

In summary, our results demonstrate that *spl-1* is an essential gene in *C. elegans* and that inhibiting *spl-1* expression results in accumulation of bioactive sphingolipid metabolites and associated severe developmental and reproductive phenotypes and indicate that the sphingolipid degradative pathway plays a conserved role in the regulation of animal development.

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